

1 **Supplementary information**

2 **Mesenchymal stem cells suppress leukemia via macrophage-mediated functional restoration**
3 **of bone marrow microenvironment**

4 Chengxiang Xia, Tongjie Wang, Hui Cheng, Yong Dong, Qitong Weng, Guohuan Sun, Peiqing
5 Zhou, Kaitao Wang, Xiaofei Liu, Yang Geng, Shihui Ma, Sha Hao, Ling Xu, Yuxian Guan, Juan
6 Du, Xin Du, Yangqiu Li, Xiaofan Zhu, Yufang Shi, Sheng Xu, Demin Wang, Tao Cheng, Jinyong
7 Wang

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9 **Supplementary Figures and Figure legends**

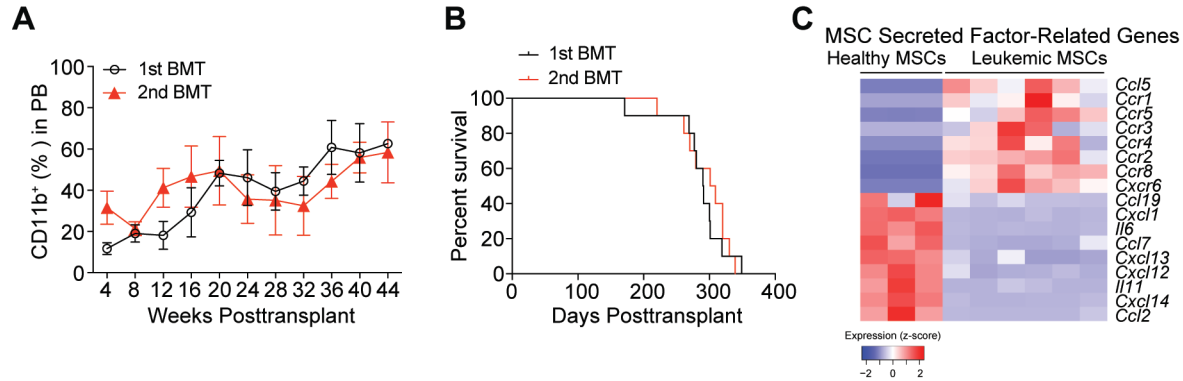


Fig. S1 Kinetics of tumor burden (CD11b⁺) and survival curves of primary and secondary leukemia-bearing mice, and the expression of soluble factors in healthy and leukemic MSCs

(A) Kinetic analysis of donor-derived myeloid cells (CD11b⁺) in PB of primary transplantation (1st BMT) and secondary transplantation (2nd BMT) recipients (mean ± SD, n = 10). **(B)** Kaplan-Meier survival of primary and secondary transplantation recipients. Kaplan-Meier survival curves of 1st BMT (black line, n = 10, Median survival = 290.5 days) and 2nd BMT (red line, n = 10, Median survival = 305 days) leukemia-bearing mice are shown. Log-rank (Mantel-Cox) test: p = 0.6105. **(C)** Heatmaps of MSC secreted factor-related genes differentially expressed between healthy MSCs (n = 3) and MSCs from leukemia-bearing mice (n = 6) (p_{adj} < 0.05, fold change > 2).

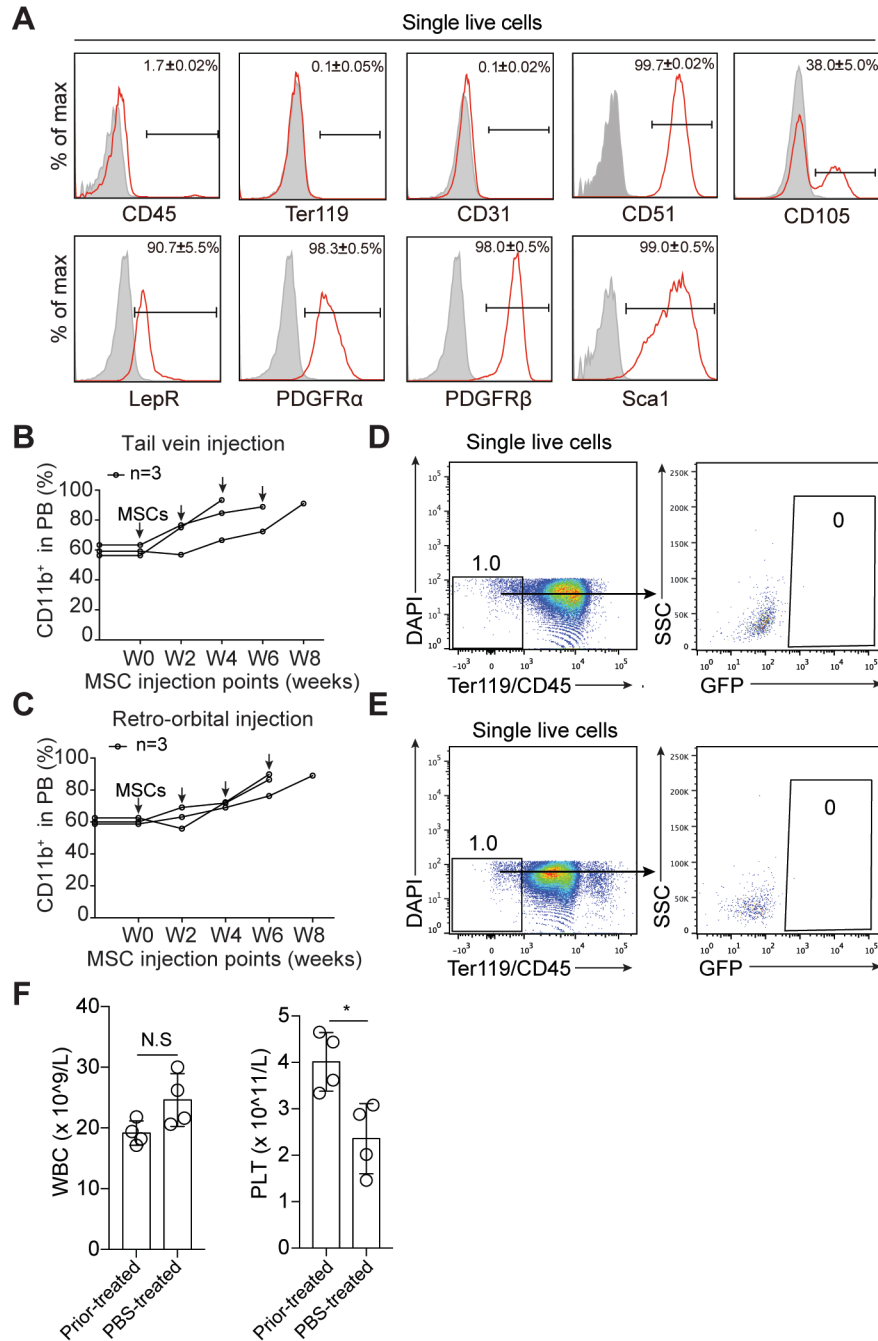


Fig. S2 Systemic delivery of donor MSCs by tail vein or retro-orbital injection and intra-BM PBS treatment fail to suppress leukemia

(A) Characterization of donor MSCs prior injection. The expanded MSCs (passage 2) were analyzed by flow cytometry. The isotype controls of each antibody were used as negative controls, as shown in the grey histograms (mean \pm SD, $n = 3$). **(B and C)** Kinetics analysis of tumor burden

27 (CD11b⁺) of MSC-treated leukemia-bearing mice by tail vein injection (B) and retro-orbital
28 injection (C). **(D and E)** Flow cytometry analysis indicated that tail vein injection (D) and retro-
29 orbital injection (E) of donor MSCs (GFP⁺) failed to home to the bone marrow of leukemia-bearing
30 mice. **(F)** Statistical analysis of white blood cells (WBC) and platelets (PLT) counts in the PB of
31 intra-BM PBS-treated leukemia-bearing mice (mean \pm SD, n = 4). Asterisk indicates *p < 0.05
32 (unpaired student's t-test (two-tailed)). N.S indicates not-significant.

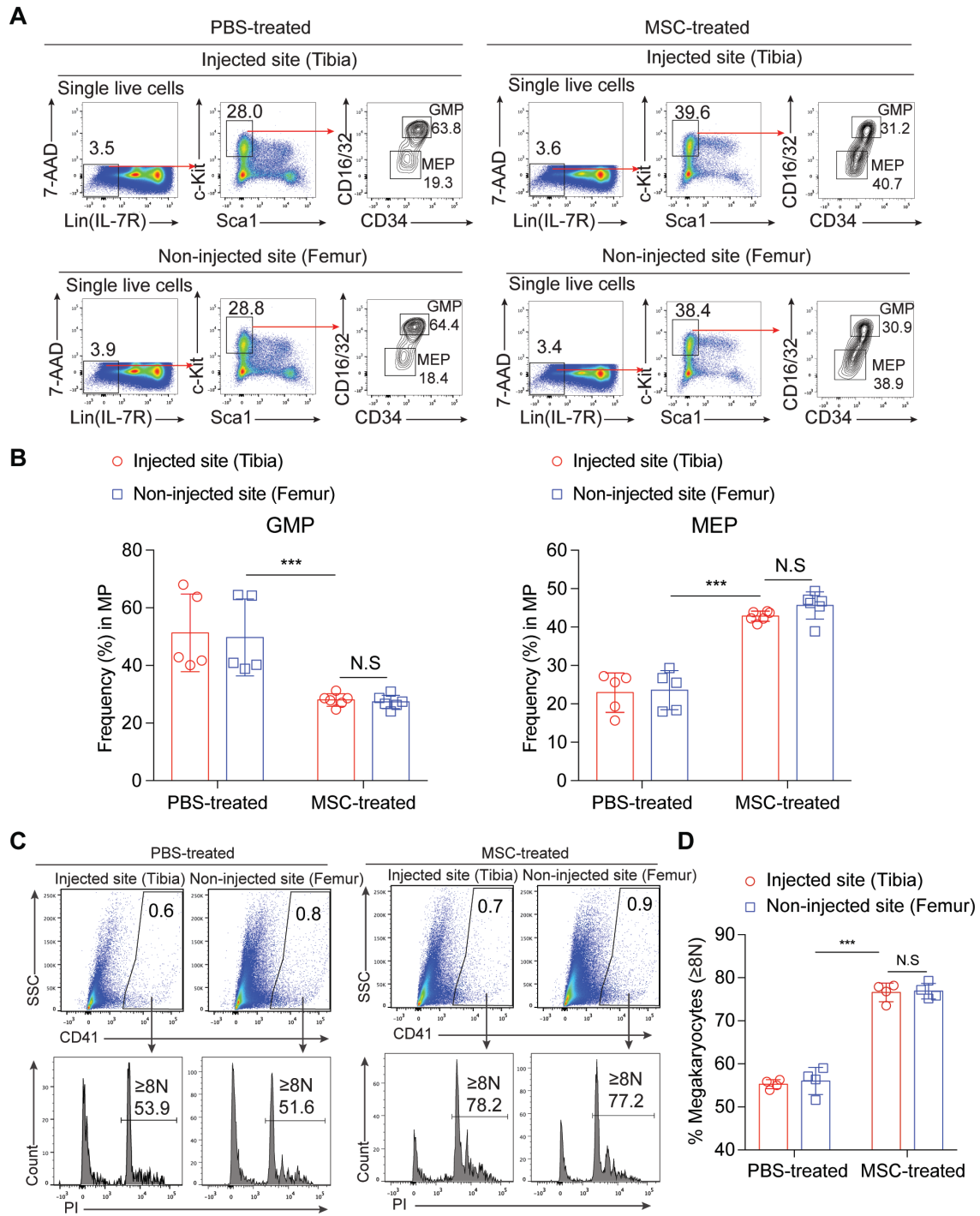


Fig. S3 Systemically re-balanced myeloid lineage progenitor cells and systemically activated megakaryocytes in MSC-treated leukemia-bearing mice

(A) Ratios of myeloid progenitor subpopulations in MSC- and PBS-treated leukemia-bearing mice. GMP (granulocyte/macrophage progenitors): Lin⁻IL-7R⁻Sca1⁻c-Kit⁺CD34⁺CD16/32^{high};

38 MEP (megakaryocyte/erythroid progenitors): Lin⁻IL-7R⁻Sca1⁻c-Kit⁺CD34⁻CD16/32⁻. **(B)**
39 Statistical analysis of myeloid progenitor components (GMP and MEP) (mean ± SD, n =5-6). **(C)**
40 Activation analysis of megakaryocytes in MSC- and PBS-treated leukemia-bearing mice.
41 Percentages of mature megakaryocytes with 8N and greater ploidy (≥8N) are shown. **(D)** Statistical
42 analysis of the percentages of mature megakaryocytes (≥8N) (mean ± SD, n =4). Asterisks indicate
43 ***p < 0.001 (one-way ANOVA). N.S indicates not-significant.

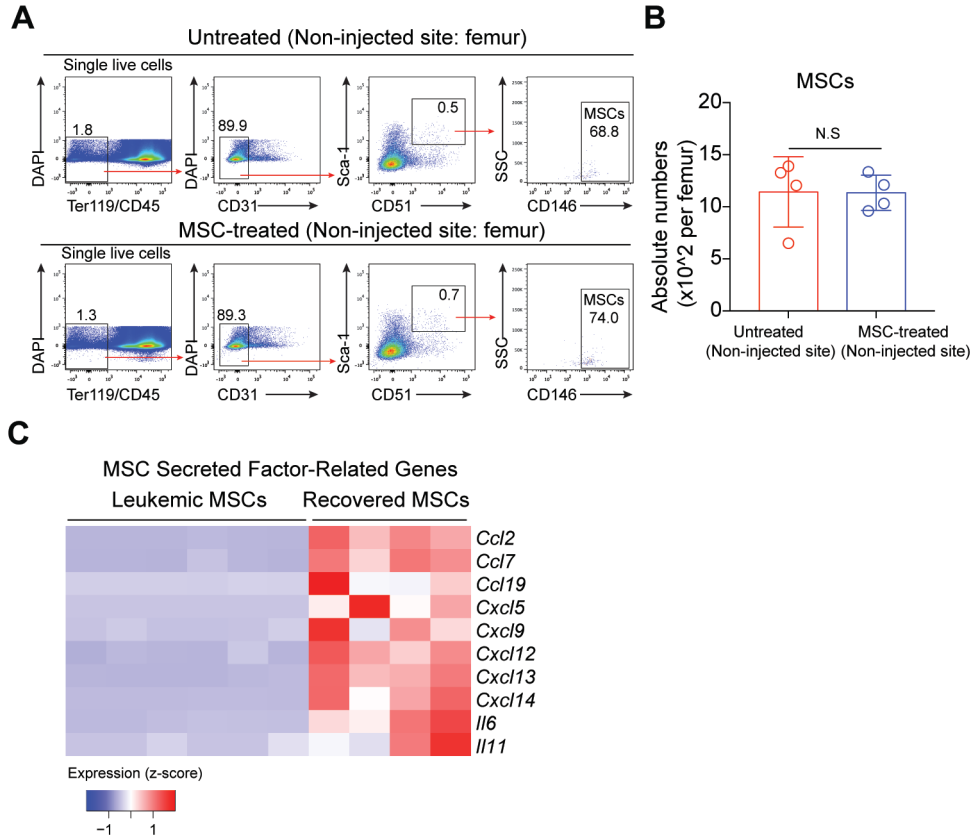


Fig. S4 No improvement in the quantity of host MSCs at the non-injected sites of MSC-treated leukemic mice and the expression of soluble factors in recovered MSCs

(A) Flow cytometry analysis of MSCs at the non-injected site from leukemia-bearing mice eight weeks post MSC treatment. MSCs are defined as Ter119⁻CD45⁻CD31⁻Sca1⁺CD51⁺CD146⁺. **(B)** Statistical analysis of the absolute numbers of MSCs in the femurs (non-injected site) from untreated and MSC-treated leukemia-bearing mice (mean \pm SD, n = 4). **(C)** Heatmaps of MSC secreted factor-related genes differentially expressed between MSCs from leukemia-bearing mice and recovered MSCs (padj < 0.05, fold change > 1.4). N.S indicates not-significant (unpaired student's t-test (two-tailed)).

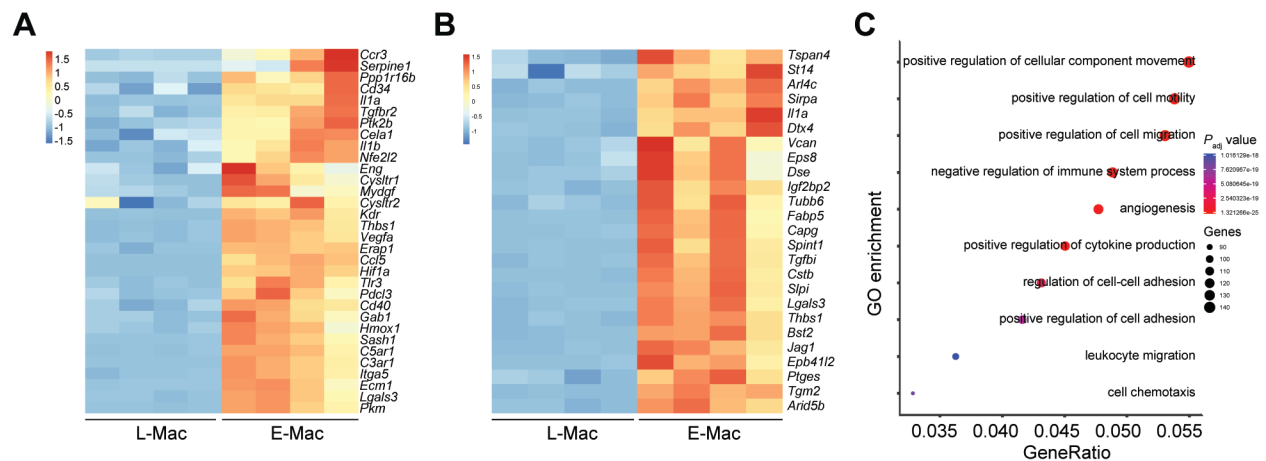


Fig. S5 Up-regulation of leading edge genes related to positive regulation of angiogenesis and cell migration pathways in MSC-reprogrammed macrophages from leukemia-bearing mice

Positive regulation of angiogenesis- (A) and cell migration-related (B) genes up-regulated in MSC-reprogrammed macrophages from leukemia-bearing mice are shown (n = 4, one per column) (a difference in expression of over 2-fold; adjusted p value, < 0.05 (DESeq2 R package)). (C) Gene ontology (GO)-enrichment analysis of the 3277 differentially expressed genes between L-Mac and E-Mac.

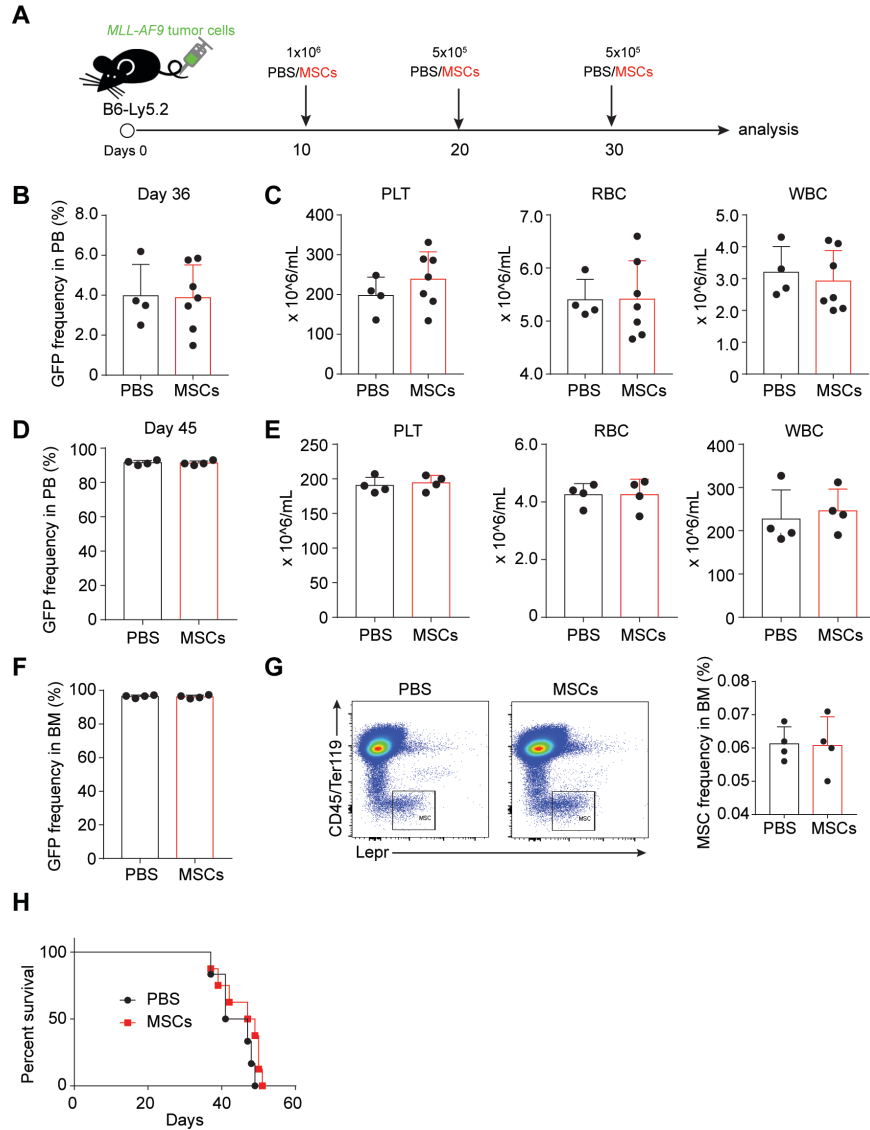


Fig. S6 MSC treatment fails to suppress AML initiated by *MLL-AF9* translocation

(A) Treatment protocol. AML cells (GFP⁺) were i.v. injected at day 0, and then the mice were intra-tibia injected with 10 μ L PBS or MSCs every 10 days from day 10. (B) Frequency of AML cells in PB at day 36. (C) PLT, RBC and WBC counts of AML-bearing mice at day 36. (D) Frequency of AML cells in PB at day 45. (E) PLT, RBC and WBC counts of AML-bearing mice at day 45. (F) Frequency of AML cells in BM at day 45. (G) Flow cytometry analysis of frequencies (right panel) of MSCs in the BM of leukemia-bearing mice at day 45. (H) Survival curve of AML-bearing mice treated with PBS or MSC. Error bars show SD, n=4-6.

Supplementary Materials and Methods

Flow cytometry analysis. Antibodies for hematopoietic lineage analysis: FITC-TER-119 (TER-119), PerCP-Cyanine5.5-CD45.1 (A20), FITC-CD45.2 (104), PerCP-Cyanine5.5-CD45.2 (104), APC-Thy1.2 (53-2.1), APC-CD3e (145-2C11), PE-CD19 (1D3), PE-Cy7-CD11b (M1/70), APC-eFluor®780-Gr-1 (RB6-8C5), FITC-CD41 (MWReg30), PE-CD61 (2C9.G3), and APC-eFluor®780-F4/80 (BM8) antibodies were purchased from eBiosciences or Biolegend. DAPI was used to exclude dead cells.

For MSC analysis, BMNC were stained with the following antibodies: APC-Ter119 (TER-119), APC-CD45 (30-F11), PE-Cy7-CD31 (WM-59), APC-eFluor®780-Sca1 (D7), PE-CD51 (RMV-7), and PerCP-Cyanine5.5-CD146 (ME-9F1) were purchased from eBiosciences or Biolegend. DAPI was used to exclude dead cells.

For myeloid progenitors staining, BM cells were stained with the following antibodies: CD2 (RM2-5), CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), Ter119 (TER-119), CD11b (M1/70), B220 (6B2), Gr1 (RB6-8C5), IL-7R (A7R34), Sca1 (E13-161.7), c-kit (2B8), CD34 (RAM34), and CD16/32 (93). DAPI was used to exclude dead cells.

For megakaryocyte maturation detection, BM cells were stained with labeled with CD41-FITC (MWReg30). Then cells were fixed using cold 70% ethanol. After washing, the fixed cells were resuspended in propidium iodide.

For platelet staining and counting, 5 µL fresh whole blood was collected. Whole blood sample was blocked with anti-mouse CD16/32, then was stained with anti-mouse CD41-FITC and anti-mouse CD61-PE at room temperature for 20 mins. Then 1 mL of cold 1% PFA solution and 50 µL absolute counting beads (C36950, Invitrogen) were added to each sample. The sample was fixed on ice for at least 30 mins.

For intracellular flow cytometry staining, BM cells were blocked with anti-mouse CD16/32 and stained with surface markers for 20 min on ice. Then cells were fixed using IC fixation buffer (88-8824-00, eBiosciences) and pulsed vortex to mix. After washing, cells were resuspended with 1X permeabilization buffer and stained with the intracellular antibody APC-Arginase 1 (A1exF5, eBiosciences) for 40 min at room temperature. Finally, the cells were resuspended with FCS buffer for analysis.

The stained cells were analyzed on LSR Fortessa (BD Bioscience), then the data were analyzed using Flowjo software (FlowJo).

Preparation of BMNC. Mice were sacrificed, and BM cells were isolated by flushing out the tibias and femurs using DPBS containing 2% FBS. The compact bones were dissected into ~2 mm fragments and transferred with 5ml of 1 mg/ml collagenase II solution into a 50 ml tube. The tubes were incubated in a shaker (< 110 rpm) at 37°C for 1-2 hours. BMNC from BM cells and compact bones were mixed and filtered through a 70 µm cell strainer (BD Falcon) to obtain a single-cell suspension.

MSC sorting. BMNC mixtures of BM and compact bones from the control mice (age-matched wild-type, CD11b⁺% in PB = 10-15%), leukemia-bearing mice (CD11b⁺% in PB = 35%-45%) and leukemia-bearing mice eight weeks post-treatment with GFP⁺ MSCs were isolated as previously described. After lysis of red blood cells, BMNC were blocked by Fc blocker, and incubated with biotin-conjugated anti-CD45 antibody and enriched by streptavidin magnetic beads (Miltenyi Biotec). The enriched CD45⁻ cells were stained with the following antibodies: APC-Ter119 (TER-119), APC-CD45 (30-F11), streptavidin-APC, PE-Cy7-CD31 (WM-59), APC-eFluor®780-Sca1 (D7), PE-CD51 (RMV-7) were purchased from eBiosciences. DAPI was used to exclude dead

117 cells. MSCs were sorted by the gating strategy defining as GFP⁻Ter119⁻CD45⁻CD31⁻Sca1⁺CD51⁺
118 using AriaII (BD Bioscience) and subsequently prepared for RNA-Seq.

119 **Mouse colony-forming unit-fibroblast (CFU-F) assay.** For analyzing the quantity of functional
120 MSCs, BMNC equivalent to 100 MSCs from each mouse were used as cell input for individual
121 wells (six-well plate). BMNC were suspended into 2 ml of mouse complete MesenCult™ medium
122 (Catalog 05513, StemCell Technology), then seeded into the individual wells. BMNC were
123 incubated at 37°C with 5% CO₂ in a humidified chamber. Half-medium change was performed on
124 day 7. After 14 days, the wells were washed once with DPBS and fixed by ice-cold ethanol, and
125 then stained with giemsa stain at RT. After washing, colonies with more than 20 spindle-shaped
126 cells per colony were counted. Three replicates of each sample were performed.

127 **Isolation and expansion of mouse MSCs.** MSCs were isolated from cell mixture of compact
128 bones and BM cells of 3-4 weeks old healthy GFP mice (n = 100), as previously reported with
129 minor modifications [1]. Briefly, the BM cavities were flushed to thoroughly deplete
130 hematopoietic cells. The compact bones were dissected into ~2 mm fragments and transferred with
131 5ml of 1 mg/ml collagenase II solution into a 50 ml tube. The tubes were incubated in a shaker (<
132 110 rpm) at 37°C for 1-2 hours. The fragments were washed three times and cultivated in complete
133 MSC culture medium (α-MEM (Gibco) supplemented with 10% FBS (Gibco) and 1%
134 penicillin/streptomycin (Invitrogen)) in a 6 cm dish. Besides, MSCs from the BM cells were sorted
135 (Ter119⁻CD45⁻CD31⁻Sca1⁺CD51⁺CD146⁺) directly into MSC culture medium. These two sources
136 of MSCs from compact bones and BM cells were mixed for further isolation and expansion. The
137 bone fragments were removed, and culture medium was replaced after three times' washing on the
138 third day. After culture for five days, the adherent cells were harvested by 0.25% trypsin's
139 digestion and passaged. The culture medium was changed every 48 hours and passaged at a split

ratio of 1:3 every 3-4 days. The expanded MSCs (Passage 2) were cryopreserved with 90% DMSO and 10% FBS in liquid nitrogen for transfusion. The cryopreserved P2 MSCs were recovered and cultured for 4-5 days, phenotypically identified, and collected in DPBS ($2.5 \times 10^7/\text{ml}$) for transfusion.

GFP-MSCs and BM macrophage co-culture assay. Short-term co-culture assay was performed, with each well containing: 1×10^5 GFP-MSCs (passage 2; healthy MSCs were isolated from GFP mice) and 2×10^6 CD11b⁺ leukemic cells sorted from leukemia-bearing mice in 2 mL culture medium of α -MEM, 10% FBS and 50 ng/ml SCF. MSCs and CD11b⁺ leukemic cells were incubated either by direct-contact culture or transwell culture for 12 hours at 37°C under 5% CO₂ in a humidified incubator. MSC-reprogrammed macrophages (CD11b⁺F4/80⁺) were sorted for RNA-sequencing or detecting the gene expression by Q-PCR.

Treatment for leukemia-bearing mice with MSC-reprogrammed macrophages. 1×10^5 MSCs were seeded into each well of six-well plates. CD11b⁺ leukemic cells were enriched from BM of leukemia-bearing mice with severe tumor burden (CD11b⁺% in PB > 60%). Then 2×10^6 CD11b⁺ leukemic cells were directly co-cultured with MSCs. After 12 hours, macrophages were sorted for transfusion. Leukemia-bearing mice with severe tumor burden were treated by intra-BM transfusion of PBS or MSC-reprogrammed macrophages from leukemia-bearing mice (E-Mac). A sequential doses of E-Mac (3.3×10^7 E-Mac/kg per dose in 20 μl PBS) were delivered into the tibia cavities of leukemia-bearing mice with two-week intervals. Analysis of platelets and CD11b⁺ cells in PB was performed monthly.

RNA-Seq and data analysis. For MSC library preparation, MSCs were sorted from wild type or leukemia-bearing mice, and recovered MSCs were sorted from leukemia-bearing mice 8 weeks post treatment with GFP⁺ donor MSCs. MSCs were sorted from two mice of each group. 1000

target cells per sample were sorted into 500 μ l DPBS-BSA buffer (0.5%BSA) using 1.5ml EP tube and transferred into 250 μ l tube to spin down with 500 g. The cDNA of sorted 1000-cell aliquots were generated and amplified as described previously [2]. The qualities of the amplified cDNA were examined by Q-PCR analysis of housekeeping genes (*B2m*, *Actb*, *Gapdh*, *Ecfla1*). Samples passed quality control were used for sequencing library preparation by illumina Nextera XT DNA Sample Preparation Kit (FC-131-1096).

For macrophages (*in vivo*) library preparation, macrophages were sorted from BM of leukemia-bearing mice before or after MSC treatment (12 hours post MSC treatment). Macrophages were also sorted after 12 hours of co-culture with MSCs. 1×10^5 target cells per sample were sorted and total RNA was extracted using the RNeasy micro kit with on-column DNase treatment (Qiagen, 74004) according to manufacture's protocol. cDNA library was constructed using VAHTSTM mRNA-seq V3 Library Prep Kit for Illumina (Vazyme, NR611) according to manufacture's protocol. The qualities of the cDNA were examined by qPCR analysis of housekeeping genes (*B2m*, *Actb*, *Gapdh*, *Ecfla1*). Samples that passed quality control were used for sequencing.

For data analysis, all libraries were sequenced by illumina sequencers NextSeq 500. The fastq files of sequencing raw data samples were generated using illumina bcl2fastq software (version: 2.16.0.10) and were uploaded to Gene Expression Omnibus public database (GSE 125029). Raw reads were aligned to mouse genome (mm10) by HISAT2 [3] (version: 2.1.0) as reported. And raw counts were calculated by featureCounts of subread [4] (version 1.6.0). Differential gene expression analysis was performed by DESeq2 [5] (R package version: 1.18.1). Unsupervised clustering analysis was performed using facotextra (R package, version: 1.0.5). Heatmaps were plotted using gplots (R package, version 3.01). GSEA was performed as described [6], and gene-

ontology (GO)-enrichment analysis were performed by clusterProfiler [7] (R package, version: 3.6.0). MSC stemness related genes and MSC osteogenesis related genes for heatmaps were from references as follows: MSC stemness-related genes [8-10] and MSC osteogenesis-related genes [8, 11]. The gene sets for GSEA were from literatures as follows: angiogenesis-related genes in macrophages [12], cell migration-related genes in macrophages (from MSigDB genesets), and secreted factors by macrophages [13, 14].

Complete blood count (CBC). For mouse samples, 100 μ l PB from each mouse was collected into 1.5 ml anticoagulation tube and diluted with the same volume of PBS, then performed complete blood count by automatic blood analyzer (Abbott, CD3700SL).

Quantitative real-time PCR. For analysis of mRNA expression levels of related genes in MSCs and CD11b⁺ leukemic cells from the co-culture assay, 1×10^5 target cells of each sample were sorted by flow cytometry using Aria II. Total RNA was extracted using the RNeasy Micro Kit (Cat NO. 74004, QIAGEN). On-column DNase digestion of the samples was performed following the manufacturer's instruction. First strand cDNA was synthesized from 100 ng of total RNA in 20 μ l final volume, using the ReverTra Ace qPCR RT Master Mix kit (FSQ-301, TOYOBO) according to the manufacturer's instructions. Real-time quantitative PCR assays were carried out in a BioRad CFX96 Real-Time PCR Detection System instrument (Bio-Rad) using standard PCR conditions. Triplicates of all reactions were performed. GAPDH gene was used as a reference for differential expression comparison. The primer sequences of all related genes are shown as below:

Gapdh (Forward (5'-3'): TGGTGAAGGTCGGTGTGAACG, Reverse (5'-3'): CAATGAAGGGGTCGTTGATGGC); *Arg1* (Forward (5'-3'): CATTGGCTTGCGAGACGTAGAC, Reverse (5'-3'): GCTGAAGGTCTCTTCCATCACC).

MLL-AF9 AML mouse model. We used a non-irradiated acute myeloid leukemia mouse model described previously [15]. *MLL-AF9* AML model mice were maintained a specific pathogen-free animal facility at the State Key Laboratory of Experimental Hematology.

Statistical analysis. Statistical analysis was performed with SPSS(SPSS v.23, IBM Corp., Armonk, NY, USA) . Normal distribution of data was tested with SPSS applying Shapiro-Wilk normality test. The data were represented as mean \pm SD. Two-tailed independent Student's t-tests were performed for comparison of two groups of data. For the analysis of three groups or more, one-way ANOVA was used, and further significance analysis among groups was analyzed by Post Hoc Test (equal variances, Turkey-HSD; unequal variances, Games-Howell). Kaplan-Meier method was used to calculate survival curves of leukemia, and Log-rank (Mantel-Cox) test was performed to compare differential significance in survival rates. P values of less than 0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

References

1. Zhu H, Guo ZK, Jiang XX, Li H, Wang XY, Yao HY, et al. A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nat Protoc* 2010 Mar; 5(3): 550-560.
2. Tang F, Barbacioru C, Nordman E, Li B, Xu N, Bashkirov VI, et al. RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nat Protoc* 2010 Mar; 5(3): 516-535.
3. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015 Apr; 12(4): 357-360.
4. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014 Apr 1; 30(7): 923-930.
5. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; 15(12): 550.

6. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005 Oct 25; 102(43): 15545-15550.
7. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 2012 May; 16(5): 284-287.
8. Freeman BT, Jung JP, Ogle BM. Single-Cell RNA-Seq of Bone Marrow-Derived Mesenchymal Stem Cells Reveals Unique Profiles of Lineage Priming. *PLoS One* 2015; 10(9): e0136199.
9. Song L, Webb NE, Song Y, Tuan RS. Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. *Stem Cells* 2006 Jul; 24(7): 1707-1718.
10. Rostovskaya M, Anastassiadis K. Differential expression of surface markers in mouse bone marrow mesenchymal stromal cell subpopulations with distinct lineage commitment. *PLoS One* 2012; 7(12): e51221.
11. Delorme B, Ringe J, Pontikoglou C, Gaillard J, Langonne A, Sensebe L, et al. Specific lineage-priming of bone marrow mesenchymal stem cells provides the molecular framework for their plasticity. *Stem Cells* 2009 May; 27(5): 1142-1151.
12. Medina RJ, O'Neill CL, O'Doherty TM, Knott H, Guduric-Fuchs J, Gardiner TA, et al. Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. *Mol Med* 2011 Sep-Oct; 17(9-10): 1045-1055.
13. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* 2014; 5: 491.
14. Cavaillon JM. Cytokines and macrophages. *Biomed Pharmacother* 1994; 48(10): 445-453.
15. Cheng H, Hao S, Liu Y, Pang Y, Ma S, Dong F, et al. Leukemic marrow infiltration reveals a novel role for Egr3 as a potent inhibitor of normal hematopoietic stem cell proliferation. *Blood* 2015 Sep 10; 126(11): 1302-1313.